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13. ABSTRACT *(Maximum 200 Words)*

This study will fully characterize the function of a BRCA1-containing protein complex, which appears in cells following DNA replication arrest using hydroxyurea. The complex, originally referred to as the S-phase specific complex and now termed the Hydroxyurea Induced Complex (HUIC), was observed in HeLa and in 293 cells. Hydroxyurea treatment of cells results in the reduction of BRCA1 content in the RNA polymerase II holoenzyme complex with a complementary increase in the HUIC. We have begun to characterize both complexes, and we find that the HUIC does not contain the repair proteins RAD50/MRE11/NBS1 as originally surmised, but does contain BRCA1 and the BARD1 polypeptides. We model that the HUIC results from the proteolytic degradation of the RNA polymerase II holoenzyme following DNA damage. Work is continuing to identify the polypeptides which compose the HUIC and to determine its role in breast cancer.

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Annual Summary Report

Award Number DAMD17-00-1-0164
PI: Natsuko Chiba, MD, PhD

Introduction

This project will identify key functions of the breast and ovarian specific tumor suppressor protein, BRCA1. Since the BRCA1 protein has been implicated in the repair of damaged DNA during the stage of the cell cycle in which the DNA is replicated, we thus proposed to study the HUIC complex we have identified. In the original proposal we had referred to the BRCA1-containing complex as the S-phase specific complex, and as discussed in the abstract, we now call it the hydroxyurea induced complex (HUIC). This complex is only seen after treating cells in S-phase, when DNA is replicated, and following hydroxyurea treatment, which may leave the DNA with gaps, which may signal a DNA repair response. In this reporting period, we have succeeded in expressing BRCA1 with an epitope tag and in partially purifying the HUIC, as outlined in Tasks 1 and 2 of the proposal. We developed a model that the HUIC results from the destruction of the RNA polymerase II holoenzyme following DNA damage. Published results describe the characterization of the HUIC and identification of domains important for BRCA1 association with the RNA polymerase II holoenzyme.

Body and key research outcomes

Task 1. Construct HA-tagged BRCA1 and compare polypeptides in holoenzyme vs HUIC

We originally proposed to tag BRCA1 in cells using a retrovirus expression vector, but this approach failed since stable expression of BRCA1 was toxic to the cells. We achieved this aim by expressing the HA-BRCA1 in 293 cells using recombinant adenoviruses. HA-BRCA1 expressed in this way faithfully recapitulated the protein complexes that contain the endogenous protein, and we were able to achieve partial purification using conventional chromatography. Surprisingly, the full length HA-BRCA1 did not generate the HUIC as did the endogenous protein, but we found that a deletion mutant HA-BRCA1(Δ775-1292) did form the HUIC complex. Using this version of the tagged BRCA1, we tested first whether certain candidate proteins were associated with the HUIC. We tested whether the DNA repair RAD50/MRE11/NBS1 proteins, which had been published to copurify with BRCA1, were present in the HUIC. We found that these DNA repair proteins were not in the HUIC complex. We have found that the HUIC contained, along with BRCA1, the protein known as BARD1. Interestingly, we have found in a separate chromatographic fraction BRCA1 associated with the DNA repair RAD50/MRE11/NBS1 proteins. Thus, the previously published observation was confirmed, but not the HUIC. These results were published and the reference is cited below as Chiba and Parvin, 2001.

In addition, we tested which of these deletion variants of BRCA1 associate with the RNA polymerase II holoenzyme. We found that the amino terminus of BRCA1 (residues 1-300) confer the strongest effect in binding to the RNA polymerase II holoenzyme. Interestingly, this same domain binds to the BARD1 protein found in the HUIC, described above. From these data, we developed a model that the RNA polymerase II holoenzyme is degraded following hydroxyurea treatment to leave the HUIC. This study is cited below as Chiba and Parvin, 2002.

Task 2. Identify and clone unknown polypeptides in HUIC.

We have made progress on this task, but have not yet completed this. By overexpressing HA-BRCA1(Δ775-1292) in cells and treating with hydroxyurea, we have succeeded in obtaining sufficient extract for protein purification. We have fractionated these extracts by Biorex70 chromatography and sucrose gradient sedimentation, as we established for the partial purification of the HUIC (Chiba and Parvin, 2001). In parallel, we have treated cells, prepared extracts, and partially purified proteins from the same cell line, except that it does not overexpress the tagged BRCA1 protein. Using these two sources of partially purified HUIC protein in parallel, we have epitope immunoaffinity purified the HUIC. We compare the precipitating bands on protein gels for the sample which contains the tagged BRCA1 to the sample which does not express tagged BRCA1. Bands which were specifically purified by this procedure were sent for identification by mass spectrometry. Our first attempt identified two known DNA repair proteins, Ku70 and Ku80. We tested for these using antibodies specific for the Ku70 and Ku80, but while the Ku70 and Ku80 specific antibodies could immunopurify these polypeptides, they did not purify BRCA1 or BARD1 in the HUIC.

New experiments are in progress to gather sufficient protein extract for a new purification of the HUIC and identify and clone the associated polypeptides in addition to BARD1.

Tasks 3 and 4. Inhibition of HUIC function and biochemical assays.

These tasks await the successful completion of task 2. We hope to begin these in this third year of support.

Reportable Outcomes: Publications resulting from DAMD17-00-1-0164:

1. Chiba N, Parvin JD Redistribution of BRCA1 among four different protein complexes following replication blockage. **J Biol Chem** 2001; *276,* 38549-38554.

2. Chiba N, Parvin JD. The BRCA1 and BARD1 association with the RNA polymerase II holoenzyme. **Cancer Research** 2002 *In press.*

Conclusions

We have characterized the BRCA1-containing protein complex, the HUIC. This protein complex is found during the S-phase of the cell cycle and following hydroxyurea treatment. It is likely that either the formation or the function of this complex is critical to the tumor suppression activity of BRCA1. Along with BRCA1, this complex contains the BARD1 protein, but it does not contain the repair proteins, such as RAD51/MRE11/NBS1 or Ku70/80. Continuing work on this project will identify other polypeptides present in the HUIC and will determine the relationship of this complex with the RNA polymerase II holoenzyme.

Appendix

See enclosed papers from this work. One reprint of Chiba and Parvin, 2001 and one pre-print of Chiba and Parvin 2002

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Redistribution of BRCA1 among Four Different Protein Complexes following Replication Blockage*

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The BRCA1 protein is known to participate in multiple cellular processes. In these experiments, we resolved four distinct BRCA1-containing complexes. We found BRCA1 associated with the RNA polymerase II holoenzyme (holo-pol), a large mass complex called the fraction 5 complex, the Rad50-Mre11-Nbs1 complex, and a complex that has not been described previously. We observed this new complex after treating cells with hydroxyurea, suggesting that the hydroxyurea-induced complex (HUIC) is involved with the response to DNA replication blockage. After hydroxyurea treatment of cells, BRCA1 content decreased in the holo-pol and the fraction 5 complex, and BRCA1 was redistributed to the HUIC. The HUIC was shown not to contain a number of holo-pol components or the Rad50-Mre11-Nbs1 complex but was associated with the BRCA1-associated RING domain protein BARD1. These data suggest that BRCA1 participates in multiple cellular processes by multiple protein complexes and that the BRCA1 content of these complexes is dynamically altered after DNA replication blockage.

Mutations in the *BRCA1* tumor suppressor gene are associated with about 4% of all breast cancers and about 50% of all familial cases (1, 2). Emerging data indicate that BRCA1 is likely to serve as an important central component in multiple biological pathways that regulate transcription, repair of DNA damage, the cell cycle, polyadenylation of mRNAs, and chromatin remodeling (3–7). It is not clear whether all of these processes are due to one biochemical mechanism or to multiple mechanisms with BRCA1 functioning in multiple protein complexes.

BRCA1 has been found to be associated with multiple polypeptides, including BARD1, which binds to the amino terminus of BRCA1 (8). Both BRCA1 and BARD1 proteins contain a RING finger motif and BRCT repeat. The BRCA1-BARD1 complex interacts with a polyadenylation factor, CstF50 (cleavage stimulation factor), suggesting a link between the regulation of polyadenylation of mRNA and DNA repair (4). BRCA1 has ubiquitin ligase activity, and, in association with BARD1, ubiquitin ligase activity is high (9–11). Direct specific interactions between BRCA1 and other polypeptides include the transcriptional regulators Pol II,[1] RNA helicase A, p53, STAT1, myc, and CtIP and repair mediators including Rad50 and BACH1 (12–21).

Several BRCA1-containing complexes have been purified using different methods. BRCA1 involvement in transcription is indicated by its association with the RNA polymerase II holoenzyme (holo-pol) and by activation of transcription by BRCA1 in cell free reactions (12, 22–24). BRCA1 is associated with the chromatin-remodeling SWI-SNF (mating type switch/sucrose non-fermenters) complex, either in association with holo-pol (23) or independent of Pol II (25). BRCA1 association with Rad50-Mre11-Nbs1 may contribute to repair of DNA damage. The BRCA1-associated genome surveillance complex contains various proteins for DNA repair, including the Rad50 complex, cell cycle check point, and DNA replication factors (26). Because BRCA1-associated genome surveillance complex is derived from a single-step immunoprecipitation (IP) from unpurified nuclear extracts, it is unclear whether it represents multiple complexes or a single complex.

BRCA1 protein dynamically changes its subcellular localization, depending on the cell cycle or whether the genome has been damaged. In S phase, BRCA1 localizes to discrete nuclear foci (27), but treatment with hydroxyurea (HU), UV irradiation, or γ-irradiation leads to dispersal of these BRCA1 foci (28). After HU and UV treatment, BRCA1 colocalizes with BARD1 and RAD51 in proliferating cell nuclear antigen-containing replication structures (29). After HU treatment or irradiation, BRCA1 forms a complex with Rad50, Mre11, and Nbs1 in discrete nuclear foci (irradiation-induced foci) (19, 20). It is unknown whether these changes in subcellular position reflect changes in BRCA1 protein complexes.

In this study, we observed BRCA1 associated with three protein complexes in asynchronously cycling cells, and BRCA1 shifted to a fourth protein complex after cells were treated with HU. These data support a concept that the multiple processes with which BRCA1 is involved reflect multiple protein complexes with which it associates.

MATERIALS AND METHODS

Cell Culture and Biochemical Purification—HeLa-S3 cells and 293S cells were passaged in suspension culture using standard procedures. About 5×10^9 cells were infected with recombinant adenovirus at a multiplicity of infection of about 1–2 plaque-forming units/cell, and cells were harvested 44 h after infection. The purification from whole-cell extracts by chromatography on a Biorex70 ion exchange matrix and sucrose gradient sedimentation have been described previously (22, 23).

Adenovirus Construction—HA epitope-tagged full-length and deleted mutant BRCA1 were inserted into AdEasy (Quantum Biotechnology,

* This work was supported by Fellowship DAMD17-00-1-0164 from the United States Army Medical Research and Materiel Command (to N. C.), Grant RPG-99-097-01 from the American Cancer Society, and pilot grants from the Elsa U. Pardee Foundation and The Concert for the Cure (to J. D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Pol II, RNA polymerase II; holo-pol, RNA polymerase II holoenzyme; HU, hydroxyurea; HUIC, hydroxyurea-induced complex; IP, immunoprecipitation; HA, hemagglutinin.

The BRCA1 and BARD1 Association with the RNA Polymerase II Holoenzyme[1]

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ABSTRACT

We have previously shown that endogenous BRCA1 and overexpressed epitope-tagged BRCA1 are present in the transcription complex called the RNA polymerase II holoenzyme (holo-pol). In this study, we further characterized BRCA1 association with the holo-pol by overexpressing deletion mutants of epitope-tagged BRCA1. We found that BRCA1-associated RING domain protein (BARD1) is a component of the holo-pol complex. Deletion of the BRCA1 NH_2 terminus, which is bound by BARD1 as well as other proteins, eliminates >98% of BRCA1 association with the holo-pol. In contrast with earlier observations, deletion of the COOH terminus of BRCA1 did not affect significantly the association with holo-pol. Immunocytochemistry of expressed full-length and deletion mutants of BRCA1 showed that the NH_2 terminus of BRCA1 is important for nuclear dot formation in S-phase. An intact BRCA1 NH_2 terminus is required for the association with holo-pol and for subnuclear localization in S-phase foci. Taken together, these data support a role for BRCA1 regulation of holo-pol function.

INTRODUCTION

The breast and ovarian cancer susceptibility gene *BRCA1* is involved in the processes of transcription, DNA repair, and ubiquitination. How BRCA1 mediates these diverse functions is unclear.

BRCA1 is a component of the RNA holo-pol[3] (1, 2). The holo-pol contains the core RNA polymerase II enzyme plus many accessory factors. BRCA1 fused to a DNA-binding domain activates transcription in cell-free systems to a similar extent as does the powerful activator, VP16 (3, 4). Many transcriptional regulators have been reported to interact with BRCA1, *e.g.*, RNA Pol II, RNA helicase A, p53, STAT1, myc, CtIP, ZBRK1, ATF family members, and estrogen receptor (3, 5–15). The presence of BRCA1 in the main mRNA synthesizing transcription machine, the holo-pol complex, and BRCA1 association with regulators of transcription highlight the importance of BRCA1 transcription function.

BARD1, the BRCA1-associated RING domain protein, has been identified as a protein that associates with the NH_2 terminus of BRCA1 (16). BARD1 protein has a RING finger motif and BRCT repeat, as has BRCA1. After DNA damage, BRCA1-BARD1 association is stimulated, and the polyadenylation of mRNA transcripts is repressed (17). BRCA1 has ubiquitin polymerase activity by itself, and the BARD1 association significantly enhances the ubiquitin polymerase activity (18–20). Specific targets of BRCA1-mediated ubiquitination have not been identified. The potential importance of ubiquitination in BRCA1 function is also suggested by the association of the NH_2 terminus of BRCA1 with a de-ubiquitinating enzyme known as BRCA1-associated protein 1 (21).

Subcellular localization of BRCA1 changes are dynamically dependent on the cell cycle or DNA damage. In S-phase of cell cycle, BRCA1 localizes to discrete nuclear foci (dots) with BARD1 and Rad51 (22–24). The protein complexes in the nuclear foci are unknown. It is unknown which BRCA1 domain is important for the structure of nuclear dots and whether BRCA1 changes of subcellular positions correlate with the holo-pol association.

In this study, we analyzed the BRCA1 association with holo-pol using deletion mutants of BRCA1. We found that BARD1 is a component of the holo-pol, and deletion of the BRCA1 NH_2 terminus severely limits the association of the mutant BRCA1 protein with the holo-pol complex. This deletion mutant also fails to form nuclear foci, leading to the suggestion that the nuclear foci might contain holo-pol, although other protein complexes associated with the BRCA1 NH_2 terminus may be responsible for the BRCA1 nuclear dot formation. Our data suggest that BRCA1 is involved in the function of Pol II in several ways via association with the holo-pol and possibly via subnuclear localization.

MATERIALS AND METHODS

Cell Culture and Biochemical Purification. 293S cells were passaged in suspension culture using standard procedures. About 5×10^9 cells were infected with recombinant adenovirus at a MOI of about 0.4–1.0 pfu/cell and cells were harvested 44 h after infection. The purification from whole cell extracts by chromatography on a Biorex70 ion exchange matrix and sucrose gradient sedimentation have been described previously (1, 2). On a Biorex70 ion exchange matrix, 600-1000 mg of whole cell extracts were fractionated. About 70% of the extract protein did not bind to the column, 5% was in the 0.3 M KOAc peak, 8% was in the 0.6 M KOAc peak, and 2% was in the 1.5 M KOAc peak. Distributions of BRCA1 on Biorex70 fractionations were estimated by the intensity of bands of immunoblot and accounting for the protein volumes of the each fraction.

Adenovirus Construction. HA-epitope tagged full-length and deleted mutants BRCA1 were inserted into AdEasy (Quantum Biotechnology, Inc.) shuttle vectors such that the *BRCA1* gene would be under the control of the cytomegalovirus promoter. Full-length HA-epitope tagged BRCA1, with the tag fused to the NH_2 terminus (HA-BRCA1) or to the COOH terminus (BRCA1-HA), were subcloned from constructs in the pcDNA3 vector (22). The 1–302 deletion was constructed by digestion of *Hin*dIII and *Eco*RI from BRCA1-HA and then ligated for insertion of the following forward (F) and reverse (R) linkers: F, 5′-AGCTTATAATGACCGGTG-3′; and R, 5′-AATTCACCGGTCATTATA-3′. The 305–770 deletion was constructed by digestion of *Eco*RI and *Kpn*I from HA-BRCA1 and then ligated for insertion of the following forward (F) and reverse (R) linkers: F, 5′-AATTCGGACCAAAGAAGAAGCGTAAGACCGGTCTGGTAC-3′; and R, 5′-CAGACCGGTCTTACGCTTCTTCTTTGGTCCACCG-3′. The 775-1292 deletion has been described previously (25). The 1527–1863 deletion mutant was generated by digestion of full-length HA-BRCA1 of pcDNA3–5′ HA-BRCA1 with *Hin*dIII and *Sac*I, and then this fragment was inserted into the AdEasy shuttle vector. With each shuttle vector construct, recombination occurred in bacteria to recover adenoviral genomic DNA with the *BRCA1* gene, and virus was recovered following transfection into 293A cells. In two of the deletion mutants, Δ305–770 and Δ775–1292, the linker that replaced the BRCA1 sequences also contained the nuclear localization sequence from the SV40 large T antigen, Pro-Lys-Lys-Lys-Arg-Lys.

Adenoviruses were prepared using standard protocols, and virus titers were determined by the $TCID_{50}$ method (Quantum Biotechnologies, Inc.). The MOI for each virus, when using 293 cells was: HA-BRCA1 (full-length, NH_2-terminal tag), 0.8 pfu/cell; BRCA1-HA (full-length, COOH-terminal tag), 1.0

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[3] The abbreviations used are: holo-pol, polymerase II holoenzyme; Pol II, polymerase II; BARD1, BRCA1-associated RING domain protein; HUIC, hydroxyurea-induced complex; IP, immunoprecipitation; MOI, multiplicity of infection; pfu, plaque-forming unit.

pfu/cell; HA-BRCA1(Δ1–302), 1.0 pfu/cell; HA-BRCA1(Δ305–770), 0.6 pfu/cell; HA-BRCA1(Δ775–1292), 0.4 pfu/cell; and HA-BRCA1(Δ1527–1863), 0.6 pfu/cell. The MOI for each virus, when using MCF-10A cells was: HA-BRCA1 (full-length, amino-terminal tag), 120 pfu/cell; HA-BRCA1(Δ1–302), 130 pfu/cell; HA-BRCA1(Δ305–770), 50 pfu/cell; HA-BRCA1(Δ775–1292), 80 pfu/cell; and HA-BRCA1(Δ1527–1863), 80 pfu/cell. These MOIs were selected for equivalent levels of HA-tagged protein expression. With 293S cells infected at an MOI of ~1, we estimated that ~70% of the cells in the culture are infected. With MCF-10A cells infected at MOIs ranging from 50 to 130 pfu/cell, all cells in the culture were infected.

IP. One hundred fifty μl of eluted protein from the Biorex70 column was immunoprecipitated with the specific antibody for human Med17 (26). Seven hundred fifty μl of binding reactions were incubated with rotation for overnight at 4°C in buffer H [20 mM Tris-OAc (pH 7.9), 1 mM EDTA, and 5% glycerol, 0.12 M KOAc, 0.1% NP40, 0.1 mM DTT, 0.2 mg/ml BSA, and 0.5 mM phenylmethylsulfonyl fluoride] in the presence of protein extract, 5 μl of antibody, and 20 μl of protein A beads. These steps are performed with or without antigenic peptide (0.1 mg/ml). With all IPs, supernatant was removed, and protein beads were then washed three times using 800 μl of wash buffer [120 mM KOAc, 20 mM Tris-OAc (pH 7.9), 0.1% NP40, 0.1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride]. For Western blot analysis, samples were subjected to electrophoresis in 5% SDS-polyacrylamide gels and immunoblotted using the indicated antibodies.

Immunocytochemistry. 293A cells were grown in DMEM supplemented with 5% FBS, 100 μg/ml penicillin and streptomycin, and infected with recombinant adenoviruses to express full-length HA-BRCA1, HA-BRCA1(Δ1–302), HA-BRCA1(Δ305–770), HA-BRCA1(Δ775–1292), and HA-BRCA1(Δ1527–1863). Cells were fixed for 10 min in PBS-buffered 3% paraformaldehyde and 2% sucrose solution, followed by 5-min permeabilization on ice in Triton buffer [0.5% Triton X-100 in 20 mM HEPES (pH 7.4), 50 mM NaCl, 3 mM $MgCl_2$, and 300 mM sucrose]. HA-BRCA1 was visualized with affinity-purified monoclonal antibody, HA.11 (Covance) as primary antibody, and FITC-conjugated secondary antibody. All images were collected by confocal microscopy.

RESULTS

Overexpression of BRCA1 Deletion Mutant Proteins. We have reported that BRCA1 could be resolved by our biochemical purification strategy into four distinct BRCA1-containing complexes: the holo-pol, a protein complex of unknown function we call the fraction 5 complex; the Rad50-Mre11-Nbs1 complex; and the HUIC (25). To characterize further the BRCA1 association with holo-pol, we prepared deletion mutants of BRCA1. The assay is to express the epitope-tagged BRCA1 deletion protein in large-scale culture and to purify the holo-pol and note the effect of the deletion. Four deletion mutants of epitope-tagged BRCA1, which nearly span the BRCA1 protein (Fig. 1*A*), were inserted into recombinant adenovirus and infected into 293S cells in suspension culture. One of these, the HA-BRCA1(Δ775–1292) has been described previously (25). Infected whole cell extracts were prepared by standard procedures (2). Expression levels of full-length BRCA1 and the four deletions of HA-tagged BRCA1 in whole cell extracts were examined by immunoblot for the HA-epitope (Fig. 1*B*). As can be seen in Fig. 1*B*, the expression levels are approximately equal for each BRCA1 protein variant. Levels of tagged BRCA1 were ~5-fold higher than endogenous BRCA1 in these extracts (data not shown). Important for analyzing protein complexes containing an overexpressed protein, cell localization data revealed no pool of cells with abnormally localized HA-BRCA1 (see Fig. 5). This suggests that protein purification data may reflect complexes with which BRCA1 is normally associated.

Whole cell extracts were chromatographed on Biorex70 ion exchange matrix, and protein fractions were eluted in washes of increasing concentration of potassium acetate (Fig. 2*A*) and analyzed by immunoblotting using antibody specific for the HA-tag and Pol II (Fig. 2*B*). The holo-pol complex and virtually all of the endogenous

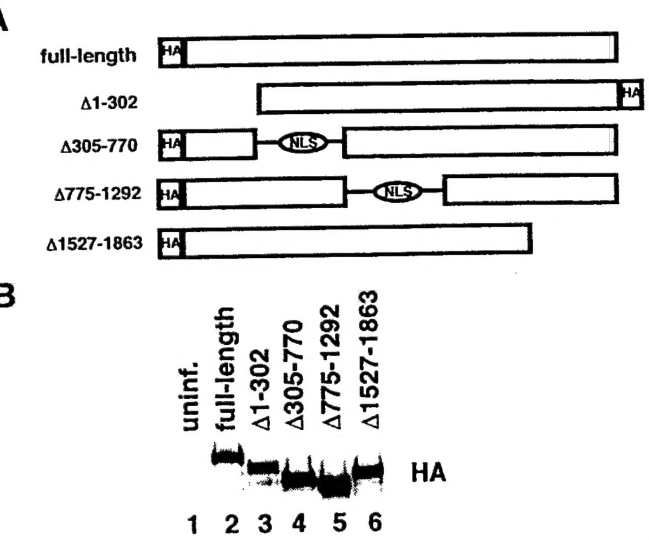

Fig. 1. Deletion mutants of epitope-tagged BRCA1 overexpressed in large scale suspension culture. *A*, design of BRCA1 deletion mutants with locations of HA-tag and locations of nuclear localization signal (*NLS*). *B*, immunoblot of 270 μg of whole cell extracts from: uninfected 293S cells (*Lane 1, uninf.*), full-length BRCA1 infected cells (*Lane 2*), and the indicated deletion mutant infected cells (*Lanes 3–6*). The blot was stained for HA-BRCA1.

BRCA1 fractionate in the 0.6 M step elution from this matrix. Unlike the tagged full-length BRCA1, the BRCA1 deletion mutants fractionated in multiple fractions from the Biorex70 matrix. Seventy % of the tagged full-length BRCA1 was fractionated in the 0.6 M KOAc fraction, and the 0.15 M KOAc flow through from this matrix contained ~10% of the total tagged full-length BRCA1 protein (factoring in the large volume of the flow-through fraction). Fractionation results were similar for the BRCA1 containing the epitope tag fused to the COOH terminus (data not shown). On the other hand, 40–50% of HA-BRCA1(Δ305–770) and HA-BRCA1(Δ775–1292) were fractionated in the 0.6 M KOAc fraction and about 40–50% in the 0.15 M KOAc flow through. In the case of the COOH-terminal deletion mutant (Δ1527–1863), 65% was eluted in the 0.15 M KOAc flow through and about 30% in the 0.6 M KOAc peak, lower than the other two internal deletion mutants. By contrast, 98% of the NH_2-terminal deletion mutant, BRCA1(Δ1–302), was fractionated primarily in the 0.15 M KOAc flow through. In this sample, endogenous BRCA1 was mainly eluted in the 0.6 M KOAc fraction, as is normally observed (data not shown). Clearly, deletion of the BRCA1 NH_2 terminus reduced the association of BRCA1 with the holo-pol-containing fraction 50-fold. The COOH-terminal deletion resulted in a minor, ~2-fold, decrease in association with the holo-pol-containing fraction.

Pol II had unchanged fractionation in these samples with the exception that expression of each BRCA1 deletion mutant resulted in some Pol II eluting in the flow-through fraction. Because it was possible that the small amount of Pol II eluting in the flow-through fraction was in fact holo-pol, we analyzed whether BRCA1 in the flow through was associated with holo-pol. This question was most critical for BRCA1(Δ1–302), which had little fractionation in the 0.6 M salt elution, where the holo-pol is typically found. Using the holo-pol-specific, affinity-purified antibody against Med17 to immunopurify holo-pol from the flow-through fraction, we found that only a small amount of the Pol II in that fraction was associated with the holo-pol (Fig. 3*A*). By contrast, BRCA1(Δ1–302), which primarily eluted in the flow through, had only background levels of binding to the holo-pol in that fraction (Fig. 3*A*). When this assay was repeated with other deletion mutants of BRCA1 in the flow-through fraction, the

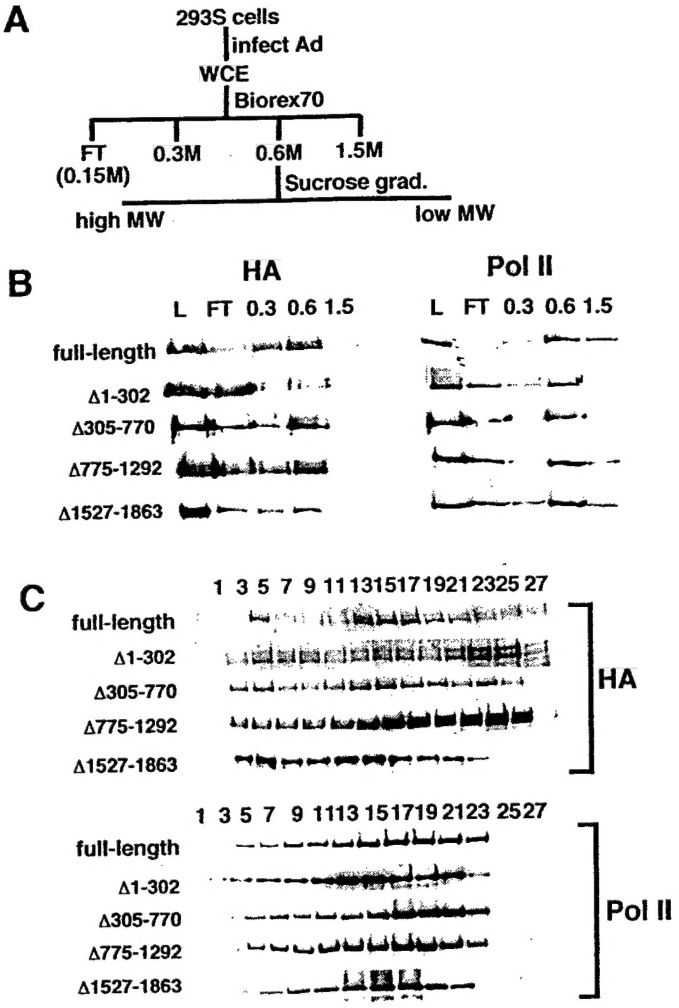

Fig. 2. Fractionations of BRCA1 deletion mutants. *A*, scheme for the partial purification of holo-pol from adenovirus-infected cell extracts. *MW*, molecular weight. *B*, immunoblots of eluted fractions from Biorex70 matrix. Blots were stained for the HA epitope (*left panel*) and the Pol II large subunit (*right panel*). *C*, immunoblots of sucrose gradient sedimentation fractions. Blots were stained for the HA epitope (*upper panel*) and the Pol II large subunit (*lower panel*).

results were similar to as seen with Pol II. Only a small amount of protein in that fraction was associated with holo-pol (data not shown). These data are most consistent with the interpretation that overexpression of BRCA1 deletion mutants alters the holo-pol complex by causing more Pol II to fractionate in the flow-through fraction.

Interestingly, Western blots for Pol II in samples containing the COOH-terminal deletion mutant (Δ1527–1863) required prolonged exposure to visualize Pol II. We infer from the last observation that BRCA1 may be involved in the turnover of Pol II.

We next fractionated the 0.6 M KOAc fractions that contain holo-pol using sucrose gradient sedimentation and analyzed by immunoblotting using antibody specific for the HA-tag and Pol II (Fig. 2*C*). The full-length protein sediments in two protein peaks, the fraction 5 complex and the holo-pol, consistent with our prior observations (25). The fractionation patterns of HA-BRCA1(Δ305–770) and HA-BRCA1(Δ775–1292) were similar, and they made three peaks of HA-BRCA1. As we described in a previous report (25), these three peaks are the >60S fraction 5 complex, the 30S holo-pol complex, and the smaller HUIC. The COOH-terminal deletion mutant (Δ1527–1863) sedimented similarly as did the full-length HA-BRCA1 in only two complexes, the fraction 5 complex and the holo-pol. Because the NH_2-terminal deletion mutant, HA-BRCA1(Δ1–302), was fractionated primarily in the 0.15 M KOAc flow through, we analyzed twice the amount of protein sample, and Western blots for HA-epitope required prolonged exposure to visualize HA-BRCA1. Although HA-BRCA1(Δ1–302) was fractionated broadly, it has three sedimentation peaks, consistent with a subset of this deletion mutant associated with the holo-pol. Clearly, most of the BRCA1(Δ1–302) protein does not cosediment with the holo-pol or the fraction 5 complex, although there is a minor peak of BRCA1(Δ1–302) that cosediments with the holo-pol in fractions 13–17. Recall that the vast majority of the expressed BRCA1(Δ1–302) did not copurify with the holo-pol on the first chromatographic step, making the results of the sedimentation profile suggest that only a little of this BRCA1 mutant associates with the holo-pol. BRCA1(Δ1–302) does cosediment in fractions 21–27, which contain the HUIC. Because we have identified BARD1 as a component of the HUIC, this is surprising for the BRCA1 mutant, which should not bind BARD1. The level of purification in fractions 21–27 was not high, indicating the likelihood that the cosedimentation of BRCA1(Δ1–302) with the HUIC was coincidental. Fractionation patterns of Pol II were consistent with previous observations of a broad peak typically centering on fraction 15. The pattern of the Pol II sedimentation was largely unchanged by the expression BRCA1 deletion mutants. The one exception was in the extract expressing

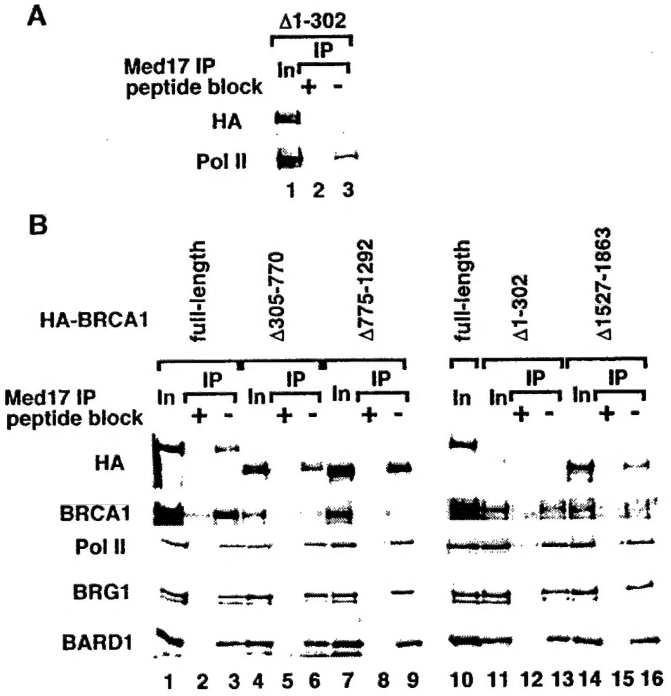

Fig. 3. Association of BRCA1 deletion mutants with the Pol II holoenzyme complex. *A*, IP by holo-pol-specific antibody directed against Med17, using 300 μl of 1.5 M KOAc Biorex70 flow through from HA-BRCA1(Δ1–302)-expressing cells as input. Ten % input protein is in the *first lane*, IP with Med17 in the presence of its antigenic peptide is in the *second lane*, and Med17 IP in the absence of blocking peptide is in the *third lane*. The *top panel* is stained using antibody specific for the HA epitope, and the *bottom panel* is stained using antibody specific for the large subunit of Pol II. *B*, IP by holo-pol-specific antibody directed against Med17, using 150 μl of 0.6 M KOAc Biorex70 eluate as inputs. All samples are evaluated in threes: 10% input protein is in the *first lane* of each trio (*Lanes 1, 4, 7, 11,* and *14*); IP with Med17 in the presence of its antigenic peptide is in the *second lane* of each set (*Lanes 2, 5, 8, 12,* and *15*); and Med17 IP in the absence of blocking peptide is in the *third lane* of each set (*Lanes 3, 6, 9, 13,* and *16*). The *top panel* is stained using antibody specific for the HA epitope. The *second panel* is stained using antibody specific for the BRCA1. The *third panel* is stained using antibody specific for the Pol II large subunit. The *fourth panel* is stained using antibody specific for the SWI/SNF subunit BRG1. The *fifth panel* is stained using antibody specific for the BRCA1-interactor, BARD1. All indicated bands migrated at positions consistent with their molecular weights.

HA-BRCA1(Δ305–770); the peak of Pol II shifted slightly to a lower molecular weight, suggesting an alteration in the polymerase content in the holo-pol complex. These results suggest that, with the exception of HA-BRCA1(Δ1–302), the deletion mutants of HA-BRCA1 copurify with holo-pol over two purification steps.

BARD1 Is a Component of holo-pol, and the NH_2-Terminal and the COOH-Terminal Regions of BRCA1 Are Both Important for Association with the holo-pol. Next, we tested whether these BRCA1 deletion mutants were truly associated with the holo-pol by IP using the holo-pol-specific antibody directed against Med17 (26). The antibody was affinity purified, and the input protein was the Biorex70 0.6 M KOAc eluate (Fig. 3*B*). All samples are evaluated in threes: 10% input protein was in the *first lane* of each trio (*Lanes 1, 4, 7, 11,* and *14*), and IP with Med17 in the presence of its antigenic peptide was in the *second lane* of each set (*Lanes 2, 5, 8, 12,* and *15*), and Med17 IP in the absence of blocking peptide in the *third lane* of each set (*Lanes 3, 6, 9, 13,* and *16*). The use of the antigenic peptide in the *second lane* of each was to control for the specificity of the IP. Immunoblots were stained using antibodies specific for the HA epitope to detect overexpressed BRCA1 (*top*), endogenous BRCA1 (*second from top*), Pol II (*third panel*), SWI/SNF subunit BRG1 (*fourth panel*), and the BRCA1-interactor BARD1 (*bottom panel*). With the full-length HA-BRCA1 (*Lanes 1–3*), we observed HA-BRCA1, Pol II, BRG1, and BARD1, all associated with the Med17. The first three of these confirmed published results (1, 2), but the identification of BARD1 associated with the holo-pol has never been demonstrated. On Biorex70 column fractionation, the total cellular BARD1 separates into the flow through and 0.6 M eluates, and it was fractionated broadly in sucrose sedimentation, including holo-pol-containing fractions. Furthermore, these fractionation patterns of BARD1 did not change by overexpressing any of the BRCA1 full-length or deletion proteins (data not shown). These results suggest that, because BARD1 can be found in protein fractions that do not contain BRCA1, not all of the BARD1 in the cells is bound to BRCA1. Rad50, although present in the input sample, was negative for immunopurification by Med17 antibody (data not shown).

We next asked whether the BRCA1 deletion mutant proteins competed with endogenous BRCA1 for binding to the holo-pol. When analyzing the full-length HA-BRCA1, it comigrated with endogenous BRCA1, making it impossible to determine which BRCA1 protein was associated with the holo-pol. The deletion mutants HA-BRCA1(Δ305–770) and HA-BRCA1(Δ775–1292) were not different from the full-length BRCA1 with regard to association with Med17. Of interest in this analysis, because these mutants migrate faster than the endogenous BRCA1, we observed that these mutants competed with the endogenous BRCA1 for binding to the holo-pol (Fig. 3, *Lanes 4–9*). This result suggests that there exist in holo-pol complex a limited number of BRCA1 binding sites, and the overexpressed BRCA1 protein competed with the endogenous BRCA1 for binding. This competition for BRCA1 binding to the holo-pol attests to the specificity of the interaction between BRCA1 and the transcription complex.

The NH_2-terminal-deleted HA-BRCA1(Δ1–302) was not enriched in the 0.6 M KOAc fraction (Fig. 2); thus, this mutant was not significantly present in the input. The small amount of HA-BRCA1(Δ1–302) present in this sample does in fact appear to be associated with the holo-pol because a weak band was present in the IP (*Lane 13*). This residual binding is consistent with our prior observation that the COOH terminus of BRCA1 is important for binding the holo-pol (2). The very low amounts of HA-BRCA1(Δ1–302) in the sample suggest, however, that there exist two domains of BRCA1 important for binding to the holo-pol, the NH_2 terminus and the COOH terminus. The NH_2 and COOH termini are partially redundant for association with the holo-pol, but much less of the total cellular BRCA1(Δ1–302) was in the holo-pol than was true for the other mutants. Endogenous BRCA1 was fractionated in the 0.6 M KOAc fraction and was not competed by low amount of HA-BRCA1(Δ1–302) for binding the holo-pol (Fig. 3, *Lanes 11–13*). These BRCA1 purification and binding data, taken together with the presence of BARD1 in the holo-pol, suggest that both the BRCA1 NH_2 terminus and the COOH terminus are very important for association with the holo-pol.

The COOH-terminal-deleted HA-BRCA1 was associated with the Med17-containing holo-pol complex, and a low amount of endogenous BRCA1 was associated with the Med17 complex. By comparing the level of competition with endogenous BRCA1 for binding the holo-pol complex, the deletion mutants HA-BRCA1(Δ305–770) and HA-BRCA1(Δ775–1292) bound to the holo-pol with higher affinity than did the COOH-terminal-deleted BRCA1. This result suggests that the COOH-terminal deletion does in fact bind to the holo-pol, possibly because of the interaction of the NH_2 terminus of BRCA1 with the holo-pol, although the amount bound with holo-pol is reduced relative to the deletion mutants HA-BRCA1(Δ305–770) and HA-BRCA1(Δ775–1292) (Fig. 3, *Lanes 14–16*). Importantly, deletion of the NH_2 terminus of BRCA1 was found to have the most profound effect on associating with the holo-pol.

The NH_2-Terminus of BRCA1 Is Important for Formation of Nuclear Dots. Because BRCA1 has known to localize in nuclear dots in the S-phase of cell cycle (27), we tested which domain of BRCA1 affects this localization. 293A cells were infected with recombinant adenoviruses to express full-length HA-BRCA1 and each deletion variant (Fig. 4). F4
Cells were stained with the anti-HA, monoclonal antibody HA.11, to visualize the expressed tagged HA-BRCA1 (Fig. 4, *A–E*) and stained with 4′,6-diamidino-2-phenylindole to show the nucleus (Fig. 4, *F–J*). Expression of full-length HA-BRCA1, HA-BRCA1(Δ305–770), HA-BRCA1(Δ775–1292), and HA-BRCA1(Δ1527–1863) and followed by probing cells using anti-HA antibody detected nuclear dots in ~10% cells. In all of these cases, we detected diffuse nuclear staining with 30–50 foci of recombinant BRCA1. Staining of cells expressing HA-BRCA1(Δ1–302) revealed only homogenous nuclear staining without foci (Fig. 4*B*). The staining patterns were specific because immunofluorescence was negative when we used anti-HA antibody preincubated with its antigenic HA-peptide for 1 h on ice (data not shown). Staining by another monoclonal anti-HA antibody, 12CA5, and anti-BRCA1(Ab-1) yielded similar results. The Ab-1 antibody detected endogenous BRCA1 in nuclear foci, even in cells expressing HA-BRCA1(Δ1–302), indicating that this mutant did not interfere with dot formation (data not shown). The results in Fig. 4 were typical for those seen on each monolayer, but accurate counts of stained cells were not possible using the 293 cells because these adhered poorly to the solid support. These results suggest that the NH_2 terminus of BRCA1, the deletion of which results in the most severe loss of interaction with holo-pol complex, is important in the formation of nuclear dots in S-phase.

Similar immunocytochemistry results were obtained using the mammary gland epithelial cell line, MCF-10A. We addressed whether there were any aberrant pools of overexpressed BRCA1 in the cells. One sign of an aberrant pool of BRCA1 would be identified by cytoplasmic accumulation. We found that the ectopically expressed HA-BRCA1 (full length) did have up to 20% of the cells with cytoplasmic staining (Fig. 5). Significantly, we observed that if the CRM1-dependent nuclear export was blocked by leptomycin B, then this protein was predominately nuclear. This suggests that the cytoplasmic BRCA1 in this experiment was not in fact aberrant, but it was regulated by the nuclear export machinery. Interestingly, the HA-BRCA1(Δ1–302), which does not copurify with the holo-pol, was

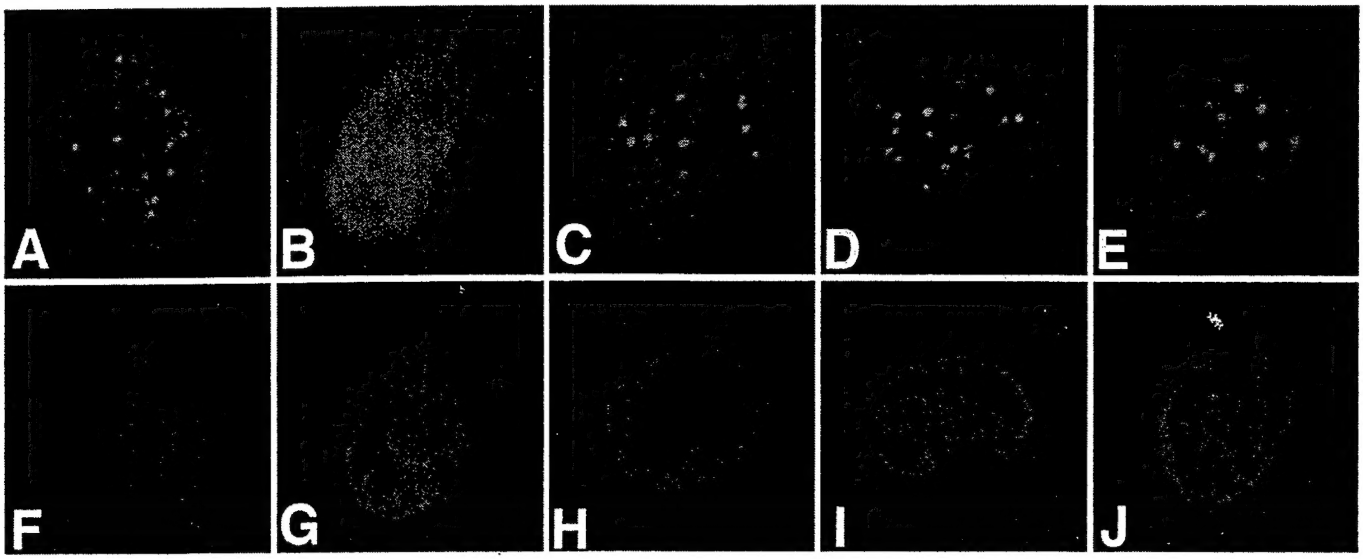

Fig. 4. The NH_2 terminus of BRCA1 is important for nuclear dot formation. 293A cells were infected with recombinant adenovirus to express full-length HA-BRCA1 (*A* and *F*), HA-BRCA1(Δ1–302) (*B* and *G*), HA-BRCA1(Δ305–770) (*C* and *H*), HA-BRCA1(Δ775–1292) (*D* and *I*), and HA-BRCA1(Δ1527–1863) (*E* and *J*). Cells were fixed and stained with anti-HA antibody (*A–E*) and 4′,6-diamidino-2-phenylindole (*F–J*), respectively.

exclusively nuclear, regardless of leptomycin B. This was likely attributable to deletion of the characterized nuclear export sequence in BRCA1 residues 89–100 (28). This result argues strongly that the failure of the BRCA1(Δ1–302) to associate with the holo-pol is not merely the result of abnormal shunting of the protein to the wrong cellular compartment but rather the deletion mutant is present in high concentration in the vicinity of the holo-pol. The other deletion mutants, which readily associate with the holo-pol, were observed to have the same subcellular distribution and response to leptomycin B, as did the full-length BRCA1 (Fig. 5*A*).

We determined the number of MCF-10A cells that form BRCA1 speckles when overexpressing tagged BRCA1 deletion proteins (Fig. 5*B*). We found that BRCA1-associated nuclear speckles were present in ~40% of the cells with nuclear staining when expressing full-length HA-BRCA1, BRCA1(Δ305–770), BRCA1(Δ775–1292), or BRCA1(Δ1527–1863) (Fig. 5*B*). The subcellular distribution and nuclear foci formation results were similar to those obtained in 293 cells, from which the protein preparations were derived, but in the latter case accurate cell counts were not possible because of the propensity of the cells to float.

DISCUSSION

Here we analyzed BRCA1 association with the holo-pol and the formation of nuclear foci by overexpressing deletion mutants of epitope-tagged BRCA1. These data showed that BARD1 is associated with the holo-pol complex and that the NH_2 terminus of BRCA1 is critical for BRCA1 association with holo-pol and for the formation of nuclear foci. The COOH terminus of BRCA1 also binds to the holo-pol, but it appears less significant than the NH_2-terminal domain.

BARD1 was found in multiple chromatographic fractions, even some fractions with undetectable BRCA1. BARD1 was associated with the holo-pol complex, BARD1 was purified by holo-pol-specific antibody, and this association was not affected by overexpression of deletion mutants of BRCA1.

The results from the various purification data are summarized in Fig. 6. The NH_2-terminal deletion mutant of BRCA1 interacts poorly with Med17-containing holo-pol complex. Instead, endogenous BRCA1 is interacting with holo-pol complex in this sample. This

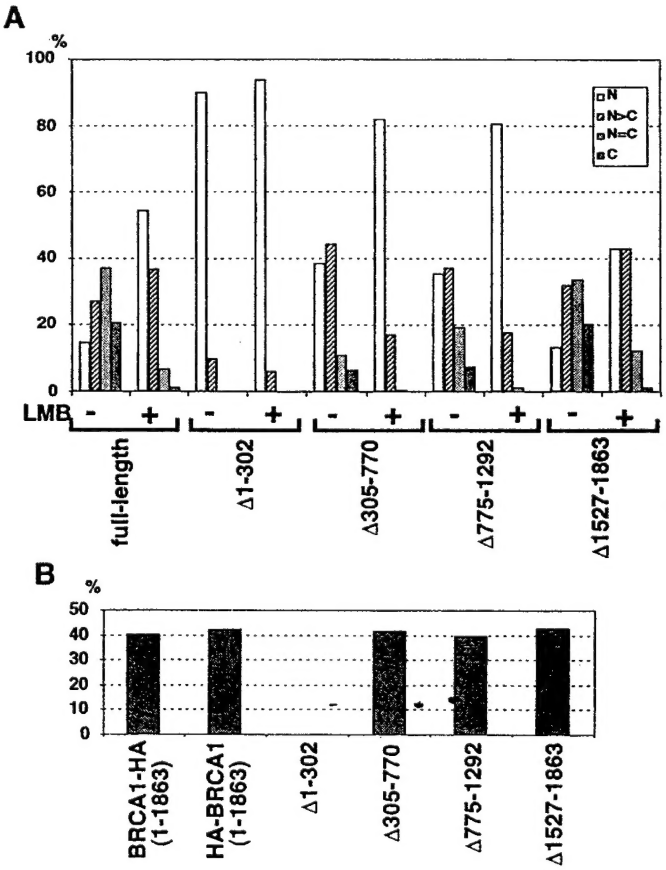

Fig. 5. Subcellular localization of BRCA1 deletion mutants in the mammary epithelial cell line, MCF-10A. *A*, 2 days after infection of MCF-10A cells with the appropriate adenovirus-expressing BRCA1 variants, cells were analyzed by immunofluorescence. About 150 cells were analyzed for each condition, and the immunofluorescence was scored as nuclear (*N*), predominantly nuclear (*N>C*), equally distributed (*N=C*), and cytoplasmic C (*C*). The nuclear export inhibitor, leptomycin B (*LMB*; 30 ng/ml), was added as indicated 8 h before staining. *B*, MCF-10A cells were treated as in *A*, without LMB, and in each sample 100 cells with predominantly nuclear staining of BRCA1 were counted for the presence of BRCA1-containing speckles.

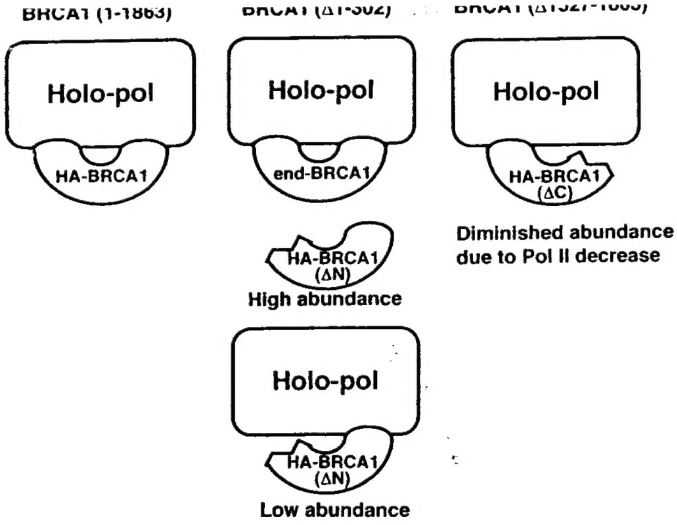

Fig. 6. Model for interaction of BRCA1 deletion mutants with Med17 complex. See text for details. The deletions of BRCA1 residues 1–302 and 1527–1863 were abbreviated ΔN and ΔC, respectively.

deletion removes the characterized binding domains for BARD1, BRCA1-associated protein 1, and part of the domain for binding p53 (6, 16, 21). It was not determined which component of the holo-pol binds to the NH_2 terminus of BRCA1, although BARD1 is a good candidate because it is in the holo-pol and it binds to this domain of BRCA1. It was surprising that deletion of the NH_2 terminus of BRCA1 resulted in a >50-fold reduction in holo-pol association, whereas deletion of the BRCA1 COOH-terminal domain resulted in a 2-fold reduction in copurification with the holo-pol. Our published experiments have demonstrated that the COOH terminus of BRCA1 can bind to the holo-pol complex (1, 2). Both domains can independently bind to the holo-pol. Deletion of the BRCA1 COOH terminus does not abrogate interaction with the Med17-containing holo-pol complex, because the BRCA1 NH_2 terminus is still present in BRCA1(Δ1527–1863). Previous results in which deletion of the COOH-terminal 11 amino acids of BRCA1 resulted in diminished binding to the holo-pol (1), probably reflect a smaller fraction of the BRCA1 association with the holo-pol. Such a possibility is consistent with our observation of a quantitative decrease in HA-BRCA1(Δ1527–1863) copurifying with the holo-pol. Alternatively, the 11 amino acid truncation could cause misfolding and thus result in a more severe effect than the larger deletion. Previous analyses of the BRCA1 COOH terminus used *in vitro* binding assays, which reveal domains that function positively to bind. By contrast, in this study, we analyzed deletion mutants in the context of the rest of the BRCA1 and not a fusion protein. The results of this study revealed that although the COOH terminus could bind to the holo-pol, deletion of the NH_2 terminus had a more profound effect on holo-pol association.

Finally, we showed that the NH_2 terminus of BRCA1 is important for formation of nuclear foci. BRCA1 localizes to nuclear foci with BARD1 in S-phase (23, 24). This same NH_2-terminal deletion, which no longer associates with nuclear foci, was at least 50-fold reduced for association with the holo-pol. It is possible that BRCA1 association with the holo-pol complex is important for formation of nuclear dots. Alternatively, the BRCA1 NH_2 terminus interacts with many proteins, and nuclear foci may result from one of these other interactions. We model that the holo-pol complex, containing BRCA1, functions in the surveillance of DNA for damage and upon encountering damaged DNA, ubiquitinates holo-pol components to recruit the proteasome, leading to a residual BRCA1-containing complex that binds to DNA repair factors (29). Our damage-surveillance model is consistent with our correlation that the NH_2 terminus of BRCA1 is important for both, binding to the holo-pol and binding with BRCA1 nuclear foci. Our prior observation that hydroxyurea treatment, which leads to halted DNA synthesis and DNA gaps, leads to a shift in BRCA1 content from the holo-pol to a new complex called the HUIC, which also contains BARD1 (25), is consistent with this concept of BRCA1 function. Perhaps the HUIC is the residual BRCA1-containing complex after the proteasome degrades many subunits of the holo-pol. Further work is aimed at testing this model for BRCA1 function and relating it to the etiology of breast cancer.

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